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ALKALOIDS FROM AUSTRALIAN FROGS (MYOBATRACHIDAE): PSEUDOPHRYNAMINES AND PUMILIOTOXINS

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ABSTRACT.—Australian frogs of the genus *Pseudophryne* contain two distinct classes of alkaloids. The pseudophrynamine class (3a-prenyl pyrrolo[2,3-*b*]indoles) are unique to this genus of frogs of the family Myobatrachidae, while the pumiliotoxin-A class (8-hydroxy-8-methyl-6-alkylidene-1-azabicyclo[4.3.0]nonanes) also occur in dendrobatid frogs of the genera *Dendrobates, Epipedobates,* and *Minyobates*, in ranid frogs of the genus *Mantella*, and in bufonid toads of the genus *Melanophryniscus*. All seven species of *Pseudophryne* examined contain both classes of alkaloids. The pseudophrynamines were the predominant class in both species (*Pseudophryne guentheri* and *Pseudophryne occidentalis*) from Western Australia, while all of the eastern species (*Pseudophryne australis, Pseudophryne bibronii, Pseudophryne coriacea, Pseudophryne corroboree*, and *Pseudophryne semimarmorata*) contained significant amounts of both pseudophrynamines and pumiliotoxins. Pumiliotoxins, in particular pumiliotoxin B, were predominant in two eastern species (*P. australis* and a southern population of *P. corroboree*), while pseudophrynamines were dominant in *P. bibronii*, four of six populations of *P. coriacea*, one population of *P. semimarmorata*, and a northern population of *P. corroboree*. Structures are proposed for several new alkaloids of the pseudophrynamine class.

Skin secretions from amphibians contain a wide range of biologically active substances (1), including lipophilic alkaloids. Most alkaloids that have been detected and characterized are from frogs of the family Dendrobatidae, where more than 200 of these have been assigned an identifying code comprised of a number (the molecular weight) followed by a letter, if necessary, to distinguish alkaloids with the same nominal mass (1,2). Dendrobatid alkaloids of the pumiliotoxin A class have also been detected in a South American toad of the genus *Melanophryniscus* (Bufonidae), in two species of Madagascan frogs of the genus *Mantella* (Ranidae, subfamily Mantellinae), and in one species of an Australian frog of the genus *Pseudophryne* (Myobatrachidae) (3). Subsequently, a pumiliotoxin-like alkaloid was detected in another species of *Pseudophryne* (4–7). Both pumiliotoxin B and its erythro diastereomer now have been identified in this species, *Pseudophryne coriacea* (8), along with a new class of alkaloids, the pseudophrynamines (3a-prenyl pyrrolo[2,3-*b*]indoles) (9).

This report documents the occurrence of pumiliotoxin-A-class alkaloids and pseudophrynamines in seven of the eleven described species of myobatrachid frogs in the genus *Pseudophryne*, and in various populations of *Pseudophryne coriacea*. Structures and tentative structures of alkaloids detected during this survey are presented in Figure 1.

EXPERIMENTAL

SOURCE MATERIAL.—Extracts of skins (MeOH unless otherwise noted) were obtained from frogs from the following localities in Australia.

Pseudophryne australis Gray (5 skins, 700 mg wet wt), Pearl Beach, New South Wales, September 1987. Extract in EtOH. Provided by Dr. H. Cogger, The Australian Museum, Sydney.

Pseudophryne bibronii Günther (1 skin, 150 mg wet wt), 11 km W. Yarrangobilly, New South Wales, March 1981. Provided by Dr. R.G. Zweifel, The American Museum of Natural History, New York.

P. coriacea Keferstein. Frogs were collected by J.W.D. and C.S. Voucher specimens are deposited with The American Museum of Natural History, New York. A. 3 skins, 400 mg wet wt, 34 km N. Childers, Queensland, January 1987; B. 2 skins, 380 mg wet wt, 9 km W. Esk, Queensland, January 1987; C.

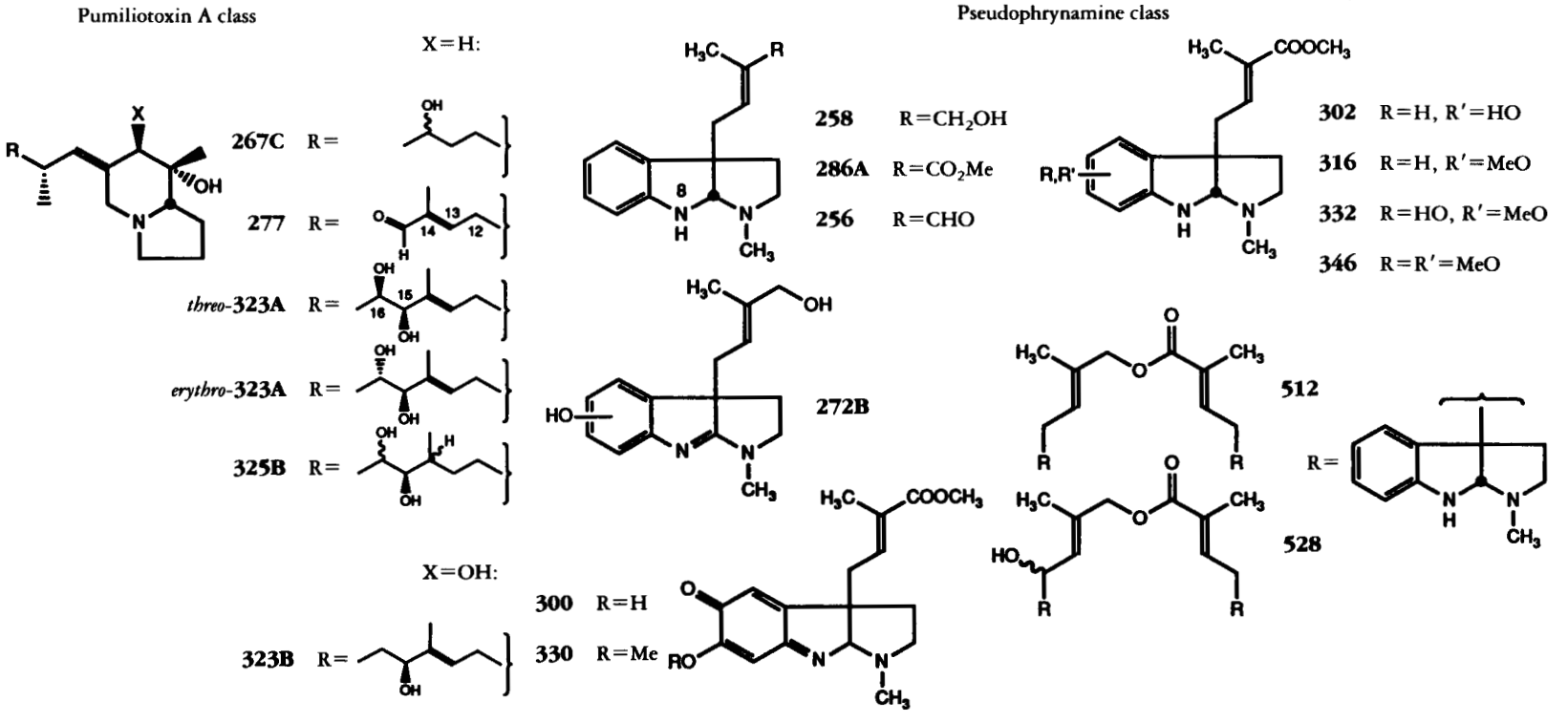


FIGURE 1. Structures for alkaloids from myobatrachid frogs of the genus *Pseudophryne*.

3 skins, 500 mg wet wt, Daisy Hill (30 km S. Brisbane), Queensland, January 1987; D. 3 skins, 420 mg wet wt, 5 km S. Nerang, Queensland, January 1987; E. 2 skins, 340 mg wet wt, 20 km E. Grafton (near Pillar Valley), New South Wales, January 1987; F. 5 skins, 1200 mg wet wt, 15 km S. Buladelah, New South Wales, January 1987.

Pseudophryne corroboree Moore. Frogs were provided to J.W.D. by Mr. Will Osborne, Department of Zoology, Australian National University, Canberra.

A. 1 Skin, 120 mg wet wt, northern form, Yarrangobilly, Kosciusko National Park, New South Wales, January 1987.

B. 5 Skins, 1200 mg wet wt, southern form, Round Mountain, Kosciusko National Park, New South Wales, January 1987.

Pseudophryne guentheri Boulenger. Skins were provided by Dr. J.D. Roberts, Department of Zoology, University of West Australia, Nedlands. Voucher specimens are in Western Australia Museum.

A. 5 Skins, 800 mg wet wt, 6 km W. junction to Walyungo National Park on Toodya Road, Western Australia, June 1987.

B. 10 Skins, 1900 mg wet wt, 40 km E. junction to Walyungo National Park on Toodya Road, Western Australia, June 1987.

C. 6 Skins, 1300 mg wet wt, 31 km S. Goomalling (Northam Road), Western Australia, June 1987.

D. 9 Skins, 1800 mg wet wt, Brookton, Western Australia, June 1987.

Pseudophryne occidentalis Parker. Skins were provided by Dr. J.D. Roberts, Department of Zoology, University of West Australia, Nedlands. Voucher specimens are in Western Australia Museum. 10 Skins, 1700 mg wet wt, 14 km N. Merredin (Wyalkatchem Road), Western Australia, June 1987.

Pseudophryne semimarmorata Lucas.

A. Provided by Dr. R.G. Zweifel, The American Museum of Natural History, New York. 1 Skin, 140 mg wet wt, Gembrook State Forest, Victoria, March 1981.

B. Lyophilized extract was provided by Dr. D. Satchell, Department of Zoology, University Melbourne, Victoria. 8 Skins, 1200 mg estimated wet wt, Victoria, 1985.

INSTRUMENTATION.—Gc analyses used He carrier gas and a 6-ft 1.5% OV-1 column on 80–100 mesh Gas Chrom Q (2 mm i.d.) or a 25-m bonded OV-1 fused silica column (Alltech, 0.25 mm i.d.) in a Hewlett-Packard Model 5890 flame ionization gas chromatograph equipped with a 3390A recorder-integrator. A Finnigan 4500 mass spectrometer with an INCOS data system was used for direct probe and gc-ms in both the chemical ionization and electron impact mode. Solid probe samples were applied to the Finnigan direct exposure probe and evaporated with a heating rate of 10 mA/sec. A Finnigan 1015 mass spectrometer and a Finnigan Model 700 Ion Trap Detector system also were used for gc-ms analysis. Thermospray mass spectrometric analyses were done by injection of MeOH extracts with a 0.05 M NH_4OAc buffer in 65% MeCN/H_2O into a Finnigan TSP46 Mass Spectrometer. Exact masses were measured by peak matching with a VG 7070F mass spectrometer or were determined by the MS Service Lab (Dr. E. Larka, Dir.), University of Minnesota. A Hewlett-Packard model 5965A FTIR instrument with a narrow band (4000–750 cm^{-1}) detector and a 59970 IRD Chem Station data system were used to record Ft-ir spectra of selected gc peaks. A fused silica capillary column [5% phenylsiloxane, 95% methylsiloxane (HP-5), 25 m × 0.32 mm] was programmed from 100° (initial time 1 min) to 250° (final time 2 min) at 10°/min to generate the total response chromatogram.

ANALYSIS OF ALKALOIDS.—Skins were cut into small pieces and ground with MeOH (or EtOH for *P. australis*) three times with at least 5 volumes of alcohol per 1 volume of skin. The combined alcohol extract was then diluted with an equal volume of H_2O and the aqueous alcohol extracted three times with one-half the volume of $CHCl_3$. The combined $CHCl_3$ layers were extracted three times with one-third the volume of 0.1 N HCl. The combined acid layers were made basic (pH>9) with 1 N aqueous NH_3 and extracted three times, each time with one-third the volume of $CHCl_3$. The combined $CHCl_3$ layers were dried over anhydrous Na_2SO_4 and evaporated in vacuo at 30°. The residue was dissolved in a volume of MeOH such that 10 μl of this alkaloid extract was equivalent to 10 mg of the original wet wt of skin.

Gc analysis was carried out as described (1,10) with alkaloid extracts equivalent to 2 mg of skin on 1.5% OV-1 packed columns using a program of 150° to 280° at 10° per min (Figures 2–4). Gc elution temperatures are given below for individual alkaloids. Chemical ionization gc-ms analyses used NH_3 or ND_3 as the ionizing gas. ND_3 reveals the number of exchangeable OH and NH protons (10). Electron-impact gc-ms and gc Ft-ir analyses with a capillary column permitted the characterization and/or identification of alkaloids. In Figure 5 we show two representative gc Ft-ir spectra of each of the alkaloid classes, pseudophrynamines (**258**, **286A**) and pumiliotoxins (**267C**, **323A**), to demonstrate the high quality of spectra obtained on submicrogram amounts of alkaloids by this relatively new gc technique. In some cases ei mass spectra were obtained with D_2O or ND_3 present and provided information on exchangeable protons in fragments as well as the molecular ion. In some instances, ei gc-ms analyses were done after acetylation. Acetylation of small portions of alkaloid extracts was carried out after removal of MeOH using a few drops

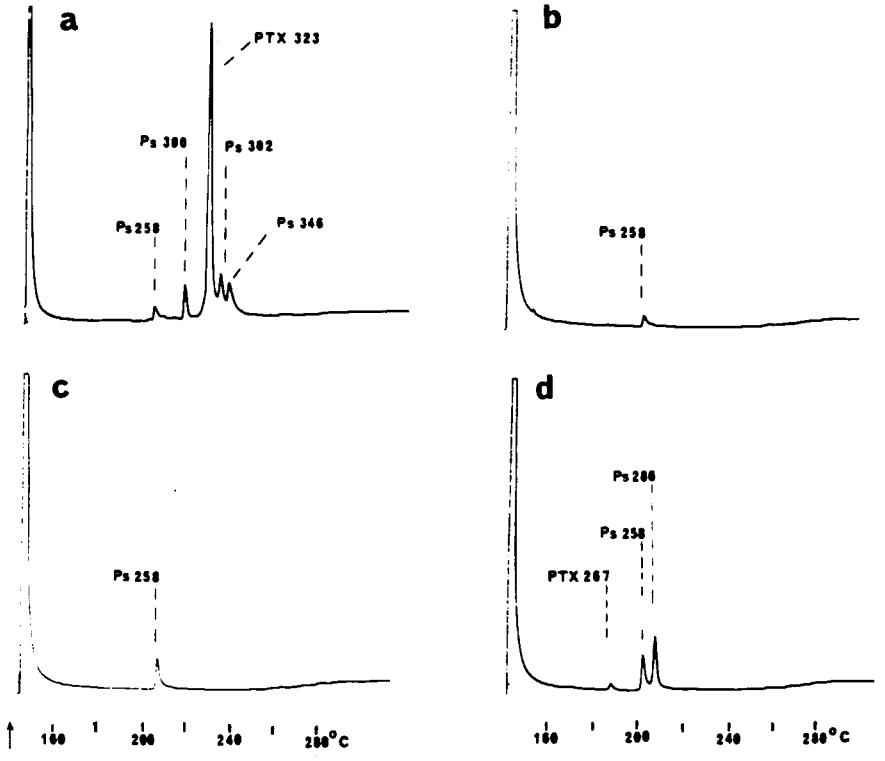


FIGURE 2. Gc profiles of alkaloids from (a) *Pseudophryne australis*, (b) *Pseudophryne guentheri* (population C), (c) *Pseudophryne occidentalis*, and (d) *Pseudophryne semimarmorata* (population B). The chromatogram was obtained with a 6-ft (2 mm i.d.) 1.5% OV-1 packed column, with a flame ionization detector and a flow rate of 30 cc/min He. A sample of 2 μl of MeOH alkaloid extract equivalent to 2 mg (wet wt) skin was injected at a column temperature of 150°. After the maximum of the solvent peak (MeOH) was passed (ca. 0.3 min) the column was heated to 280° at 10° per min. Alkaloids were identified by gc-ms analysis.

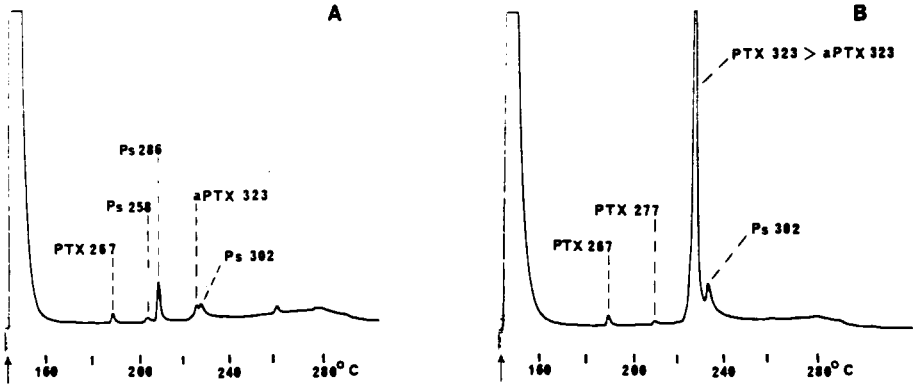


FIGURE 3. Gc profiles of alkaloids from *Pseudophryne corroboree*, populations A and B. See legend to Figure 2 for details.

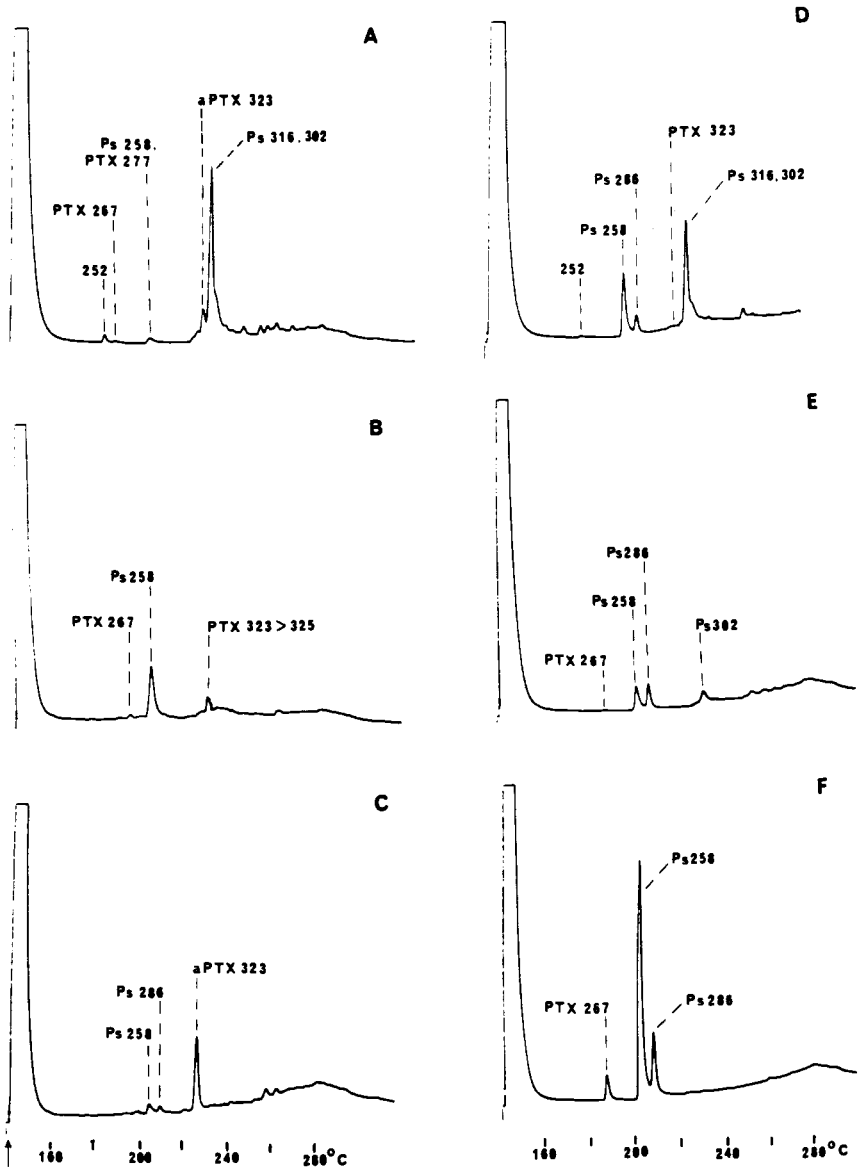


FIGURE 4. Gc profiles of alkaloids from *Pseudophryne coriacea*, populations A–F. See legend to Figure 2 for details.

of Ac_2O-pyridine (1:1) at room temperature for 2 h. Solvents were removed in vacuo and the residue redissolved in MeOH. Hydrogenation of alkaloid extracts with a Pd/C catalyst was as described (2). Thermospray ms analyses allowed for the detection of high molecular weight alkaloids of the pseudophrynamine class, which do not gas chromatograph satisfactorily (9). Such analyses provided a semi-quantitative estimate of the relative amounts of all alkaloids in each extract (data not shown). Thermospray mass spectral analysis with 0.05 M NH_4OAc buffer in 65% MeOD/D_2O revealed the number of exchangeable hydrogens in the alkaloids.

Alkaloids were also analyzed by tlc [Si gel, $CHCl_3$-MeOH (9:1)] with detection by iodine vapor or, in the case of pseudophrynamine alkaloids, the modified Ehrlich's reagent (0.1% *p*-dimethylaminocinnamaldehyde in 1 N HCl).

PROPERTIES OF ALKALOIDS.—The following section contains an assigned number and, where needed, an identifying letter in bold face for each alkaloid, the empirical formula (those in quotations have not been confirmed by high resolution ms), an R_f on Si gel [$CHCl_3$-MeOH (9:1)], an elution temperature

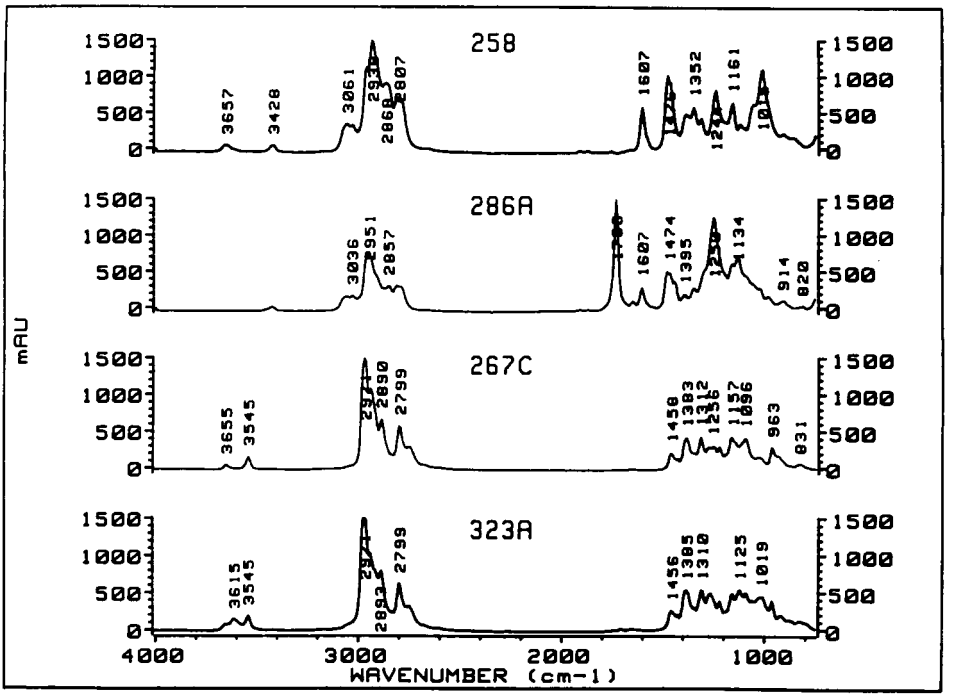


FIGURE 5. Ft-ir spectra for pseudophrynamines **258** and **286A** and pumiliotoxins **267C** and **323A**.

on packed column gc (see above), the eims with prominent peaks and other data including the derivative formed on catalytic hydrogenation, the number of hydrogens exchangeable with deuterium, ir spectral data when available, and the occurrence as a major, minor, or trace alkaloid (Tables 1 and 2) in the *Pseudophryne* species [see Daly *et al.* (1) for further details and for similar data on other alkaloids from amphibians]. Abnormal hydrogenation behavior was noted for the principal pseudophrynamine alkaloid **258** (see Discussion). Accordingly, we have omitted any hydrogenation data for this class of alkaloids.

Pumiliotoxin A class.—**267C**. $C_{16}H_{29}NO_2$; 0.28; 190°; *m/z* 267 (16), 194 (12), 166 (100), 84 (18), 70 (75); H_2-derivative; 2D; ir 3640, 3545, 2983, 2798, 2760 cm^{-1} (Figure 5). For further properties see Daly *et al.* (3). Major: One population *P. semimarmorata*. Minor: One population each *P. coriacea, P. corroboree, P. semimarmorata*. Trace: *P. bibronii*; four populations *P. coriacea*; one population *P. corroboree; P. occidentalis*. Detected as trace alkaloid in fractions from MeOH extracts of 130 skins *P. guentheri* (May 1987, Western Australia). Not detected in dendrobatid frogs. An earlier report of an isomer **267D** in *P. semimarmorata* (3) was not confirmed and appears to have been in error. Structure see Figure 1.

277. "$C_{17}H_{27}NO_2$"; 0.61; 206°; *m/z* 277 (2), 206 (12), 194 (48), 193 (58), 176 (15), 166 (20), 163 (65), 84 (24), 70 (100); H_6-derivative; 1D; ir 3541, 1703, 1645 cm^{-1}. Trace: *P. australis*; one population each *P. coriacea, P. corroboree*. Present as trace alkaloid only in extracts, including some of dendrobatid frogs (unpublished results), that contain relatively large amounts of pumiliotoxin B [**323A**]. Structure see Figure 1.

threo-**323A**. Pumiliotoxin B; $C_{19}H_{33}NO_3$; 0.17; 230°. For other properties of this "dendrobatid alkaloid" see Daly *et al.* (1). Major: *P. australis*; one population *P. corroboree*. Minor: Two populations *P. coriacea*; one population *P. corroboree*. Trace: Two populations *P. coriacea; P. occidentalis; P. semimarmorata*. Structure see Figure 1; ir see Figure 5.

erythro-**323A**. $C_{19}H_{33}NO_3$; 0.17; 230°. Trace: *P. corroboree*. Has been isolated from extracts of *P. coriacea* (166 skins, 1972–1974, Queensland) (8,9). Gc, tlc, and ms properties nearly identical to *threo*-**323A**. Distinguished by capillary gc separation of di-*O*-acetates and other derivatives (8). Structure see Figure 1.

323B. $C_{19}H_{33}NO_3$; 0.20; 228°. An allopumiliotoxin. For further properties of this "dendrobatid alkaloid" see Daly *et al.* (1). Major: One population *P. coriacea*. Trace: *P. corroboree*; one population each *P. coriacea, P. semimarmorata*. Structure see Figure 1.

325B. $C_{19}H_{35}NO_3$;—; 228°. For other properties of this "dendrobatid alkaloid" see Daly *et al.* (1). Trace: One population *P. coriacea*. Tentative structure: A 13,14-dihydro **323A** (Figure 1).

TABLE 1. Alkaloids from Myobatrachid Frogs of the Genus *Pseudophryne*.

Species and population	Total amount[a]	Alkaloid																
		Pumiliotoxin A class[b]						Pseudophrynamine class[b,c]										Others[b]
		267C	277	*threo*-323A	*erythro*-323A	323B	325A	258	286A	300	302	316	330	332	346	512	528	252B
Pseudophryne australis	++		+	+++				++		++	++	+	+	+	++			
Pseudophryne bibronii	+	+						+++	++									
Pseudophryne coriacea																		
A	++	+	+	++		+		++	+		+++	++	++	++	+	+		++
B	+	+		++				+++			++			+			+	
C	+	+				+++	+	++	++		+	+	++	++		+		
D	++			+				+++	++		++	++	++	++		+	+	+
E	+	+						+++	+++		++			+		+	+	
F	++	++		+				+++	++	+[d]			+			++	++	
Pseudophryne corroboree																		
A	++	++		++	+	+		+	+++		++					++	++	
B	+++	+	+	+++	+	+			+		++		+	+		+	+	
Pseudophryne guentheri																		
B>C>A,D[e]	+							+++										
Pseudophryne occidentalis	+	+		+				+++				+	+	+				
Pseudophryne semimarmorata																		
A	+	+++		+		+		++	+							+		
B	+	++		+				+++	+++		+					+	++	

[a]Estimated total amount of alkaloids per 100 mg skin: +++, > 150μg; ++, 50–150 μg; +, < 50 μg.

[b]Alkaloids are designated by mol wt in boldface type, followed where necessary by a code letter to distinguish from other alkaloids of the same nominal weight. +++, present as a major alkaloid (actual amount per mg skin may vary from low amounts where total alkaloid content is low to 100 μg/100 mg skin where total alkaloid content is high; see footnote a and Figures 2–4); ++, present as a minor alkaloid; +, trace alkaloid. Analysis based on gc, gc-ms, direct probe cims, and thermospray ms.

[c]For further trace alkaloids of the pseudophrynamine class see Table 2.

[d]Probably an isomer of the alkaloid in *P. australis*.

[e]Relative total amount in populations A–D (see text).

TABLE 2. Other Trace Alkaloids of the Pseudophrynamine Class from Myobatrachid Frogs of the Genus *Pseudophryne*.

Species and Population	Alkaloid[a]								
	256	**272A**	**272B**	**286B**	**302**[b]	**524**	**526**	**540**	**542**
Pseudophryne coriacea									
A					+	+			
B					+	+	+		
C					+	+	+	+	
D			+			+	+	+	
E			+		+	+	+	+	+
F	+	+		+					
Pseudophryne corroboree									
A		+			+				+
B					+			+	+
Pseudophryne guentheri									+
Pseudophryne occidentalis			+						+
Pseudophryne semimarmorata									
A			+						
B			+	+					

[a]+, Present as a trace alkaloid.

[b]An isomer of **302** (Table 1) with only one exchangeable hydrogen.

Pseudophrynamine class.—**256**. "$C_{16}H_{20}N_2O$";—; ca. 204°; *m/z* 256 (22), 228 (10), 227 (10), 199 (10), 185 (28), 173 (100), 171 (20), 156 (10), 144 (28), 130 (95), 110 (25), 109 (30); 1D; ir 2980, 2944, 2808, 1703, 1608, 1478, 1240 cm^{-1}. Trace: One population *P. coriacea* (F). A minor component in one chromatographic fraction from *P. coriacea* (166 skins, 1972–1974, Queensland). Tentative structure see Figure 1.

258. Pseudophrynaminol: $C_{16}H_{22}N_2O$; 0.30; 206°; *m/z* 258 (25), 185 (18), 173 (100), 130 (90); 2D; ir 3657, 3432, 3060, 3040, 2937, 2866, 2802, 1607, 1477, 1353, 1244, 1162, 1015 cm^{-1} (Figure 5). For further properties see Spande *et al.* (9). Major: Four populations *P. coriacea; P. guentheri; P. occidentalis*; one population *P. semimarmorata*. Minor: *P. australis; P. bibronii*; two populations *P. coriacea*; one population *P. semimarmorata*. Trace: One population *P. corroboree*. Structure see Figure 1.

272A. "$C_{17}H_{24}N_2O$";—; 204°; *m/z* 272 (50), 255 (12), 199 (27), 187 (80), 144 (100), 143 (23), 130 (18); eims (ND_3) 273 (63), 255 (15), 199 (20), 187 (80), 144 (100); 1D. Trace: One population *P. coriacea*. Tentative structure: *N*(8)-methyl derivative of **258**.

272B. "$C_{16}H_{20}N_2O_2$";—; 208°; *m/z* 272 (50), 255 (15), 199 (33), 187 (54), 144 (100), 143 (25); 2D. Trace: Two populations *P. coriacea*; one population *P. corroboree; P. occidentalis; P. semimarmorata*. Tentative structure, see Figure 1.

286A. $C_{17}H_{22}N_2O_2$; 0.53; 211°; *m/z* 286 (38), 199 (20), 185 (32), 173 (100), 157 (62), 156 (52), 130 (97); 1D; ir 3430, 3060, 3040, 2951, 2802, 1736, 1607, 1475, 1251, 1134 cm^{-1} (Figure 5). Formerly **286**, becomes **286A**, due to addition of **286B** (see below). For further properties see Spande *et al.* (9). Major: One population each *P. coriacea, P. corroboree, P. semimarmorata*. Minor: *P. bibronii*; three populations *P. coriacea*. Trace: One population each *P. coriacea, P. corroboree, P. semimarmorata*. Structure see Figure 1.

286B. "$C_{17}H_{22}N_2O_2$";—; 211°; *m/z* 286 (<1), 199 (100), 173 (7), 156 (10), 130 (7), 70 (50); 0D; ir 2950, 2794, 1754 (unconjugated ester), 1555, 1456, 1190, 1160 cm^{-1}. Trace: One population each *P. coriacea, P. semimarmorata*. Tentative structure: A double bond isomer of **286A**.

300. "$C_{18}H_{24}N_2O_2$";—; 219°; *m/z* 300 (100), 269 (23), 241 (40), 240 (43), 225 (10), 213 (20), 198 (16), 187 (47), 185 (30), 170 (19), 159 (25), 106 (34); 0D. Minor: *P. australis*. Another isomer [*m/z* 300 (78), 269 (22), 195 (20), 187 (100), 144 (75), 122 (25), 85 (57); 0D] appears to be present as trace alkaloid in one population of *P. coriacea*. Tentative structure: A quinonimine (Figure 1). Gc detection of this pseudophrynamine alkaloid (see also **330**) was difficult on capillary columns, while it is readily detected on a packed column.

302. $C_{17}H_{22}N_2O_3$;—; 234°; *m/z* 302 (66), 300 (24), 190 (35), 189 (100), 173 (20), 146 (30); 2D. Major: One population *P. coriacea*. Minor: *P. australis*; three populations *P. coriacea; P. corroboree*. Trace: One population each *P. coriacea, P. semimarmorata*. Tentative structure: An aryl hydroxy analogue of **286A**

(Figure 1). It is also accompanied by a trace isomer **302'** with only one exchangeable hydrogen in some *P. coriacea* populations and in *P. corroboree* (Table 2).

316. "$C_{18}H_{24}N_2O_3$";—; 232°; *m/z* 316 (90), 215 (22), 203 (100), 188 (37), 174 (16), 160 (85), 146 (18); eims (D_2O) 317 (68), 216 (18), 204 (100), 189 (27), 161 (85), 147 (12); 1D. Minor: Two populations *P. coriacea*. Trace: One population *P. coriacea; P. australis; P. occidentalis*. Tentative structure: An aryl methoxy analogue of **286A** (Figure 1).

330.$C_{18}H_{22}N_2O_4$; 0.61; 237°; *m/z* (direct probe) 330 (100), 302 (45), 296 (90), 282 (32), 243 (32), 217 (30), 215 (30), 199 (25), 189 (87); 0D. Minor: Three populations *P. coriacea*. Trace: *P. australis*; one population each *P. coriacea, P. corroboree, P. occidentalis*. Red color. Tentative structure: A methoxyquinonimine, occurring as an oxidation product from **332** (Figure 1).

332. $C_{18}H_{24}N_2O_4$;—; 239°; *m/z* 332 (40), 282 (10), 219 (100), 217 (30), 189 (25), 176 (35), 161 (15); 2D. Minor: Three populations *P. coriacea*. Trace: *P. australis*; two populations *P. coriacea*; one population *P. corroboree, P. occidentalis*. Tentative structure: A hydroxy methoxy analogue of **286A** (Figure 1). Two isomers detected by gc Ft-ir: ir (I) 3657, 3430, 3030, 2953, 2805, 1736, 1655, 1494, 1450, 1255, 1170, 804 cm^{-1}; ir (II) 3596, 3010, 2952, 2855, 2800, 1736, 1618, 1497, 1306, 1248, 1200, 1136, 824 cm^{-1}.

346. "$C_{19}H_{26}N_2O_4$";—; 235°; *m/z* 346 (60), 233 (100), 218 (22), 190 (60), 175 (25), eims (ND_3) 347 (47), 234 (100), 219 (20), 191 (30), 176 (18); 1D. Minor: *P. australis*. Trace: One population *P. coriacea*. Tentative structure: A dimethoxy aryl analogue of **286A** (Figure 1).

512. Pseudophrynamine A; $C_{32}H_{40}N_4O_2$; 0.38;—(not detected by gc-ms analysis); *m/z* (direct probe) 512 (56), 456 (23), 455 (13), 340 (55), 338 (100), 273 (20), 241 (40), 211 (17), 197–199 (20–23), 182–185 (22–25), 173 (80), 172 (60), 144 (20), 130 (38); 2D. For further properties see Spande *et al.* (9). Minor: One population each *P. coriacea, P. corroboree*. Trace: Four populations *P. coriacea*; one population *P. corroboree; P. semimarmorata*. Structure see Figure 1.

524. $C_{33}H_{40}N_4O_2$;—;— (not detected by gc-ms analysis); *m/z* (direct probe) high resolution 524.3100; calcd for $C_{33}H_{40}N_4O_2$, 524.3151; 0D. Trace: Five populations *P. coriacea*. Tentative structure: an *N*(8)-methyldehydro derivative of **512**.

528. "$C_{32}H_{40}N_4O_3$";—;— (not detected by gc-ms analysis); *m/z* (direct probe, less volatile component in mixture with **512**) 528 (10), 472 (5), 356 (15), 354 (25), 173 (100), 130 (95); 3D. Minor: One population each *P. coriacea, P. corroboree, P. semimarmorata*. Trace: Three populations *P. coriacea*; one population *P. corroboree*. Tentative structure see Figure 1.

In addition, other analogues of pseudophrynamine A (molecular ions 526, 540, 542) were detected by thermospray ms and by direct probe cims (NH_3) (Table 2).

Other alkaloids.—**252B**. "$C_{14}H_{24}N_2O_2$";—; 185°; *m/z* 252 (1), 221 (5), 126 (100); 1D (*m/z* 126 does not contain the exchangeable hydrogen, while *m/z* 221 does). Minor: One population *P. coriacea*. Trace: One population *P. coriacea*. Tentative structure: An alkaloid analogous to the dendrobatid alkaloid **252A** [formerly **252**; see Tokuyama *et al.* (11)] but with the hydroxy group in another part of the structure.

RESULTS AND DISCUSSION

Alkaloids of the pseudophrynamine class and pumiliotoxin-A class were detected in skin extracts from the seven species of frogs of the myobatrachid genus *Pseudophryne* that were examined (Figures 2–4). The pseudophrynamine class occurred in all species and populations, while the pumiliotoxin A class occurred in all species, but not at detectable levels in four populations of *P. guentheri* (Table 1). Alkaloids have as yet been detected only in the following families and genera of amphibians: Bufonidae, *Bufo* (morphine) (12), *Melanophryniscus* (pumiliotoxin-A class) (3); Dendrobatidae, *Dendrobates, Epipedobates, Minyobates, Phyllobates* [for revision of the family see Myers (13)] (batrachotoxins, histrionicotoxins, indolizidines, pumiliotoxin-A-class, decahydroquinolines, gephyrotoxins, piperidines, pyrrolidines, calycanthine-chimonanthine, azatricyclododecenes, and structurally undefined alkaloids) (1, 14); Myobatrachidae, *Pseudophryne* (pumiliotoxin-A class, pseudophrynamines) (3, 8, 9, and this paper); Ranidae (subfamily Mantellinae), *Mantella* (histrionicotoxins, indolizidines, pumiliotoxin-A-class, decahydroquinolines, and structurally undefined alkaloids) (3); Salamandridae, *Salamandra* (samandarines) (14, 15). The presence of pumiliotoxin-A-class alkaloids in diverse families of frogs, including the genus *Pseudophryne* of the myobatrachid family, is remarkable and seems most likely to be due to independent evolution of the

biosynthetic enzymes (3). The myobatrachid frogs of the genus *Pseudophryne* are unique in the development of the capability of producing the pseudophrynamines.

No alkaloids were detected in representative species from another six genera of myobatrachid frogs: *Adelotus brevis, Cyclorana australis, Cyclorana brevipes, Heleioporus albopunctatus, Heleioporus eyrei, Heleioporus inornatus, Heleioporus psammophilus, Mixophyes schevillii, Notaden melanoscaphus, Notaden nichollsi, Uperoleia lithonada*, and *Uperoleia nyolegi*. No alkaloids were detected in the following Australian hylid frogs: *Litoria albuguttata, Litoria lesuerii, Litoria peroni, Litoria rothi, Litoria rubella*, and *Nyctimystes tympanocrystis*.

The distribution of alkaloids among the species and populations of *Pseudophryne* is given in Tables 1 and 2. Alkaloids were previously reported from *P. corroboree* (16) and tentatively were proposed to be samandarine-like based on chromatographic properties. The present study provides no indication of any samandarine-like alkaloids in *P. corroboree* or any other species of *Pseudophryne*, and it appears unlikely that samandarines occur in such frogs. Estimates of relative amounts are from gc-ms analyses as in Figures 2–4 and from thermospray analyses. Identification is based on gc-ms comparison with known compounds (Figure 1). Tentative structures for additional alkaloids in the pseudophrynamine class (Figure 1) are based on mass spectra and other properties as documented in the Experimental section.

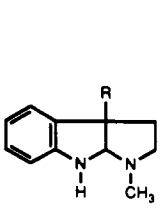
Catalytic hydrogenation, while extremely useful in characterizing dendrobatid alkaloids, was observed to yield possibly confusing data when applied to the pseudophrynamine class. While compound **286A** is slowly reduced with a 5% Rh/Al_2O_3 catalyst (1–2 atm H_2) to the expected dihydro product [**1**, two diastereomers, 45:55, with identical mass and ir spectra, *m/z* 288 (38), 173 (46), 144 (90), 130 (100); ir 1755 cm^{-1}], compound **258** undergoes a rapid hydrogenolysis to **2** and **3** followed by reduction to **4**. A sample of a *P. coriacea* extract (population F), which contained chiefly **258** and **286A**, after hydrogenation (1 h) gave a 3:2 mixture of hydrogenolysis (*m/z* 242) and hydrogenolysis-reduction (*m/z* 244) products (15% unreacted **258**, 80% unreacted **286A**). The hydrogenolysis product consisted of a major isomer **2** [14 parts, *m/z* 242 (25), 185 (25), 173 (50), 144 (15), 130 (100)] that was accompanied by a minor isomer **3** [1 part, *m/z* 242 (25), 185 (55), 173 (32), 144 (86), 130 (100)]. After 3 h, **4** is the sole product of hydrogenation. The ready hydrogenolysis of the **258** compound by this catalyst is unexpected, since we had observed previously that the minor hydrogenolysis reactions encountered during reduction of pumiliotoxin B with PtO_2 or Pd/C did not occur with Rh/Al_2O_3 (unpublished results).

A remarkable hydrogenolysis with Rh/Al_2O_3 is observed in the reduction of the **277** aldehyde where, in addition to the expected hexahydro derivative [*m/z* 283 (1), 110 (35), 84 (100), 70 (30)], roughly equivalent amounts of an *m/z* 253 product, identical to the hydrogenation product of pumiliotoxin **251D**, also result. This may arise from rearrangement, during reduction, of the 13-14 double bond to the 12-13 position, then hydrogenolysis of the hydroxymethyl group at C-14, and finally reduction of the double bonds.

The absolute stereochemistry of pseudophrynamines is unknown. However, pseudophrynamine **258** has strong negative extrema at 242 and 295 nm in the cd spectrum (9), suggesting the same configuration as (−)-physostigmine.

Among the pseudophrynamines, one is unique in being red in color. This alkaloid occurred in several populations of *P. coriacea* (Table 1), but it was apparently lost during alumina chromatography of extracts from a large mixed sample of *P. coriacea* skins (3). It may be related to rubreserine, the red pigment formed by oxidation of physostigmine (17,18).

The identification of erythro and threo isomers of pumiliotoxin B and analogous al-

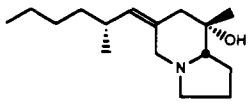


1 R= CH_2–$CH(CH_3)$–CO_2CH_3

2 R= CH_2–CH=$C(CH_3)_2$

3 R= CH_2–$CH(CH_3)$–CH_2

4 R= CH_2–$CH(CH_3)_2$



251D

kaloids of the pumiliotoxin-A class in *Pseudophryne* is based on gc-ms analysis of the alkaloids and of derivatives as previously described (8). The usual isomer of pumiliotoxin B detected in *Pseudophryne* is the threo isomer, previously isolated from New World dendrobatid frogs (1) and now shown to be the only isomer (unpublished results) in frogs of this family (*Dendrobates arboreus, Dendrobates auratus, Dendrobates histrionicus, Dendrobates lehmanni, Dendrobates pumilio, Dendrobates speciosus, Epipedobates tricolor, Minyobates bombetes*). The alkaloid extract from the Madagascan ranid frog *Mantella aurantiaca* contained mainly *threo*-pumiliotoxin B and a smaller amount of the erythro isomer [unpublished results, see Daly *et al.* (3)]. The alkaloid extract from the Brazilian bufonid toad *Melanophryniscus moreirae* contained small amounts of *threo*-pumiliotoxin B [unpublished results, see Daly *et al.* (3)].

Earlier pharmacological studies on partially purified extracts of *P. coriacea* obtained from mixed samples of 166 skins from sites designated as Bald Mountain and Boonah (Queensland) during the period 1974–1976 indicated the presence of a very active pumiliotoxin-B-like alkaloid (4–7). Attempts to identify a derivative or analogue of pumiliotoxin B in purified fractions have not been succesful (8,9). Instead only pumiliotoxin B and its erythro isomer were identified with certainty (8). Extracts from 494 skins of a collection of *P. coriacea* obtained in January 1987 from the populations A–F yielded only pumiliotoxin B (see below). However, it seems possible that certain populations of *P. coriacea* may contain a highly active analogue of pumiliotoxin B, in addition to pumiliotoxin B itself. This conclusion is based in part on the data of Table 3, where the number of "pumiliotoxin-B equivalents" in several species and populations of *Pseudophryne* are compared. It is also true that the "pumiliotoxin-B equivalents" in the earlier collections of *P. coriacea* (4) conducted in 1974–1976 at Bald Mountain and Boonah are far in excess of what can be accounted for relative to the amounts of pumiliotoxin B that ultimately were identified (Table 3). Furthermore, although combined extracts from various populations of *P. coriacea* collected in January 1987 contain lower levels of "pumiliotoxin-B equivalents" than extracts from the 1974–1976 collections, these levels still significantly exceed the levels of pumiliotoxin B detected by gc analysis (Figures 2–4) and the amounts of pumiliotoxin B isolated by alumina chromatography. Thus, only 20 mg of pumiliotoxin B was isolated from 494 skins (estimated wet wt 250 g, comprising population A, 23 skins; B, 10 skins; C, 69 skins; D, 258 skins; E, 16 skins: F, 118 skins), while bioassay in a variety of systems indicated the presence of 70–105 mg of "pumiliotoxin-B equivalents" (Table 3). The presence in the extract either of a highly active analogue of pumiliotoxin B or of a substance that enhances the activity of pumiliotoxin B is clearly indicated, but as yet no physical evi-

TABLE 3. Comparison of Pumiliotoxin-B Equivalents by Pharmacological and gc-ms Analysis in *Pseudophryne* Populations and Species.

Species and Population	Collection State,[a] Year (Number of skins)	Pumiliotoxin-B Equivalents	
		Pharmacological Activity (relative to *Pseudophryne coriacea* 1974–1976 = 100)[b]	Gc-ms Analysis (relative to *Pseudophryne corroboree* B 1987 = 100)[c]
Pseudophryne australis	NSW, 1974 (2)	7–8[d]	—
	NSW, 1987 (5)	—	80
Pseudophryne bibronii	QLD, 1966–88 (10)	2–4[d]	—
	QLD, 1972 (27)	1.5–2[d]	—
	NSW, 1981 (1)	—	<1
Pseudophryne coriacea[b]	QLD, 1974–76 (370)	100[d]	—
A	QLD, 1987 (3)	16	≲5
B	QLD, 1987 (2)	12	≲5
C	QLD, 1987 (3)	10	≲10
D	QLD, 1987 (3)	4	≲5
E	NSW, 1987 (2)	4	<1
F	NSW, 1987 (5)	10	≲2
A–F	QLD, NSW, 1987 (494)	≲13[e]	≲8
Pseudophryne corroboree	NSW, 1967 (12)	100[b]	—
A	NSW, 1987 (1)	—	≲5
B	NSW, 1987 (5)	—	100
Pseudophryne occidentalis	WA, 1987 (10)	—	<1
Pseudophryne nicholsi	WA, 1973 (12)	25[d]	—
Pseudophryne guentheri	WA, 1973 (105)	<0.3[d]	—
A–D	WA, 1987 (30)	—	≪1
Pseudophryne semimarmorata A,B	Vict., 1981–85 (9)	—	<1

[a]NSW, New South Wales; QLD, Queensland; WA, Western Australia; Vict., Victoria.

[b]The reference value of 100 corresponds to 7–12 mg pumiliotoxin-B equivalents per gram (dry wt) skin by bioassay (4).

[c]The reference value of 100 corresponds to ca. 1 mg pumiliotoxin-B equivalents (pumiliotoxin B plus allopumiliotoxin **323B**) per gram (wet wt) skin. One gram wet wt is approximately equivalent to 0.3 gram dry wt.

[d]Data from Erspamer *et al.* (4).

[e]Total pumiliotoxin B from 494 frogs (populations A–F, see text) is estimated by gc analysis to be 20 mg. Pumiliotoxin-B equivalents are estimated by pharmacological assays to be 70–105 mg.

dence for significant amounts of substances other than pumiliotoxin B in the purified fraction has been obtained.

The toad *Melanophryniscus moreirae* contained a pumiliotoxin **267C** ($C_{16}H_{29}NO_2$, Figure 1), which had not been detected previously in dendrobatid frogs (3). However, it has now been shown to occur in trace amounts in many *Pseudophryne* (Table 1). Earlier gc-ms analysis of extracts of *P. semimarmorata* had indicated the presence of an isomer of **267C**, which differed from **267C** only in having an apparent decreased retention time on gc. This apparent isomer was designated **267D** (3). It had the same R_f value as **267C** on tlc (3). It also appeared to occur in *Mantella aurantiaca* (3). However, the $C_{16}H_{29}NO_2$ alkaloid detected in all of the present extracts of *Pseudophryne* is identical in packed and capillary gc retention time to **267C** from *Melanophryniscus*. In addition, the mono-*O*-acetate derivatives of **267C** and the *Pseudophryne* alkaloid are also identical in gc-ms properties. Attempts to reevaluate the earlier extracts of *P. semimarmorata* (population A) and of *Ma. aurantiaca* (3) led to the conclusion that these extracts now contain only one $C_{16}H_{29}NO_2$ alkaloid identical in gc retention time to pumiliotoxin **267C** from *Melanophryniscus* (3). Whether the apparent presence of a shorter retention time isomer **267D** in earlier analyses was in error or whether an alkaloid **267D** has transformed in storage to **267C** during 4 years is uncertain, but none of the current frog alkaloid extracts of *Pseudophryne* contain a $C_{16}H_{29}NO_2$ alkaloid with a retention time different from that of **267C**.

The pumiliotoxin **277** (Figure 1) corresponds to the aldehyde that would be formed through oxidative cleavage of the 15,16-diol of pumiliotoxin B. It occurs as a trace alkaloid in both *Pseudophryne* (Table 1) and in some dendrobatid frogs (unpublished results). Whether it is an artifact formed by oxidation during fractionation of pumiliotoxin B, which it always accompanies, or is a natural compound is unknown. It can be prepared from pumiliotoxin B by oxidation with MnO_2 (19).

Allopumiliotoxin **323B** occurs rarely in *Pseudophryne* (Table 1) but has been previously reported from many dendrobatid frogs (1) and from *Melanophryniscus* and *Mantella* (3). It is a major alkaloid in *P. coriacea*, population C.

Finally, one alkaloid with a mol wt of 252 ("$C_{14}H_{24}N_2O_2$") was detected in extracts as a trace constituent in certain *Pseudophryne*. The mass spectrum of this alkaloid, to be designated **252B** (Table 1) with a base peak at *m/z* 126 ("$C_6H_{10}N_2O$") is reminiscent of dendrobatid alkaloid **236** (11), which also affords a base peak at *m/z* 126. It perhaps is a hydroxy regioisomer of alkaloid **252** (now designated **252A**) previously isolated from dendrobatid frogs (11).

The alkaloid profiles in *Pseudophryne* frogs may prove to be of some systematic value in delineation of relationships in this group, which often readily "hybridizes" where populations come in contact and which contains species whose validity is in question, such as the *P. semimarmorata/dendyi/bibronii* complex and *P. coriacea* complex. The latter may consist of three different species (G. Ingram, personal communication). In this regard, it should be noted that populations A, C, and D are the only populations of *P. coriacea* to contain the red alkaloid; i.e., western populations from Queensland. Also, the most southern population F from New South Wales is the only population of *P. coriacea* whose yellow pigments remained in the H_2O/MeOH phase after partitioning with $CHCl_3$; i.e., they, but not other populations, probably contain very low levels of carotenoids. The skin of *P. corroboree* has been shown to contain both pterin and carotenoid pigments (20). The remarkable variability of alkaloids in populations of *P. coriacea* is illustrated in Figure 4. The alkaloid profiles are also quite distinct for two populations of *P. corroboree* (Figure 3). These populations are very distinctive in appearance, with the northern population (A) being predominately black with yellow markings, while the southern population (B) is predominately yellow with black markings.

Alkaloid profiles in the four populations of *P. guentheri* are virtually identical, differing only in absolute amounts of a single pseudophrynamine alkaloid, namely pseudophrynaminol **258** (Table 1). Population C, which contained the highest amount of alkaloids, has been illustrated (Figure 2). Populations A and D contained about ⅕ the amount present in population C, while population B contained about ½ the amount present in population C. Alkaloids of the pumiliotoxin-A class were not detected in these 10-skin samples of *P. guentheri*. Pumiliotoxin **267C**, however, was detected in fractions from a 130-skin sample of this species (skins provided to V. Erspamer by Dr. J.D. Roberts, University of Western Australia). Two populations of *P. semimarmorata* have been examined. An earlier analysis of population A was based on one skin only, and pseudophrynamine **258** was not identified because it co-chromatographed with C_{18} fatty acid methyl esters, which were thought to be "alkaloids of unknown nature, with apparent molecular weights of 311, 313, and 315" [see Daly *et al.* (3)] determined by chemical ionization with NH_3. We now realize these ions result from NH_4+ adducts to C-18 fatty acid methyl esters of molecular weights 294, 296, and 298. On reexamination, the alkaloid extract from *P. semimarmorata* population A shows the first peak to emerge on gc to be pumiliotoxin **267C** [not **267D** as previously reported by Daly *et al.* (3)], the second, smaller peak to be **258**, contaminated with some fatty acid methyl esters, and finally the third and smallest peak to be a mixture of allopumiliotoxin **323B** and pumiliotoxin B [**323A**] [see Figure 7 in Daly *et al.* (3)]. Small amounts of pseudophrynamine **286A** were also detected. Population B of *P. semimarmorata* differs mainly in the relative amounts of the alkaloids with **286A>258≫267C>323A**. In population A the relative amounts were **267C>258≫286A~323B~323A**.

It may be noted (Table 1) that certain populations of *P. coriacea* (D, E, F) and one of *P. corroboree* (A) have proportionally more of pseudophrynamines than pumiliotoxins while other populations of *P. coriacea* (A, C) and one of *P. corroboree* (B) have an inverse relationship. This may be fortuitous only. However, it seems possible that the pseudophrynamines could serve as biosynthetic intermediates in the synthesis of the pumiliotoxins where one or two isoprene units are evidently incorporated. It should be stressed that pseudophrynamines have not been detected in any of the dendrobatid frogs or in bufonid toads where pumiliotoxins are also detected and hence are not mandatory intermediates.

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